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13. ABSTRACT *(Maximum 200 Words)*

We purposed to characterize a regulatory domain on p53 and identify kinase(s) that may be involved in the regulation. Since 7/1/97, we constructed a set of deletions to disrupt the potential regulatory domains of p53 on Gal4-p53(1-160) and on full-length p53. Using these mutants we have obtained the following results:

1. Mutants Gal4-p53N(Δ92-109), Gal4-p53N(Δ117-128) and Gal4-p53N(Ser117/127Ala) had little effect on the putative inhibitory effect.
2. TPA stimulation inhibited the transcriptional activities of Gal4-p53N(Δ92-109) and Gal4-p53N(Δ117-128), but did not affect Gal4-p53(1-92).
3. Mutants p53(Δ92-109), p53(Δ117-128), p53(S117/127A) and p53(S117/127D) significantly lost their ability to activate transcription in vivo.
4. Mutants p53(Δ92-109), p53(Δ117-128) and p53(S117/127A) retained their abilities to bind to DNA, but p53(S117/127D) not.
5. MAP-kinase is involved in the degradation of mutant p53 protein.

In conclusion, we have shown that two serine residues 117 and 127 may play a role in stabilization of p53 protein levels. Further experiments will be conducted to confirm these results. We have also showed that MAP-kinase is involved in the degradation of mutant p53 protein. Our findings suggest a role for MAPK in the degradation of mutated form of p53 protein and in cell differentiation. These results were published as a JBC paper

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INTRODUCTION

A putative domain may be present within p53 involving its regulation. Further evidence of a regulatory domain is derived from a comparison of p53 with two oncogenes, c-jun and c-fos. Amino acids 93-160 from p53 show a significant homology to c-jun and c-fos inhibitory domains, especially to c-jun's δ region. The c-jun δ region has been found to play an inhibitory role by interacting with a member of the MAPK family, JNK. Once bound to c-jun, JNK is capable of phosphorylating serine residues 63 and 73. Two conserved serine residues within p53's potential regulatory domain may also be involved in phosphorylation and regulation. Therefore, we hypothesized that p53 serine residues 117 and 127, homologous to c-jun serine residues 63 and 73, may be phosphorylated by JNK or another MAPK family member. Specifically, we proposed:

I. Construct Gal4-p53(Δ92-109) and Gal4-p53(Δ117-128) and study their transcription activities using a transient transfection assay.

II. Assay if JNK or another MAPK family member is involved in regulating p53 transcription activity.

III. Construct point mutants on serine residues 117 and 127.

IV. Study phosphorylation of p53 regulatory domain by JNK or other potential MAPK family member kinase(s).

BACKGROUND

p53 exerts its tumor suppression function by inducing growth arrest and apoptosis. The biochemical activity of p53 that is required for this relies on its ability to bind to specific DNA sequences and to function as a transcription factor. The importance of the activation of transcription by p53 is underscored by the fact that the majority of p53 mutations found in tumors are located within the domain required for sequence-specific DNA binding. Therefore, it is clear that this activity is critical to the role of p53 in tumor suppression.

Previous work by our group suggests a possible regulatory domain may be present within p53. Supporting preliminary data used Gal4-p53 constructs for deletion analysis of p53's transcription activity. Based on transient transfection assays, the region of p53 spanning residues 1-92 shows high transactivation activity. Extension of the transcriptionally active residues 1-92 with residues 93 to 160 resulted in a reduction in transcription by a factor of 100.

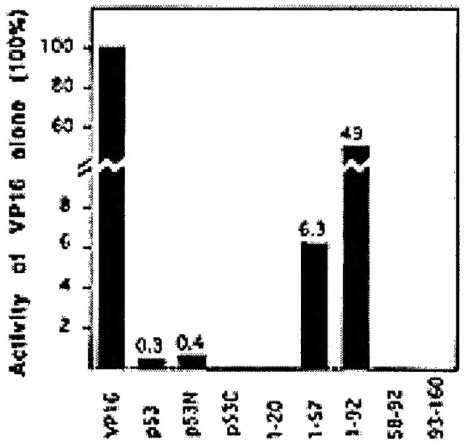
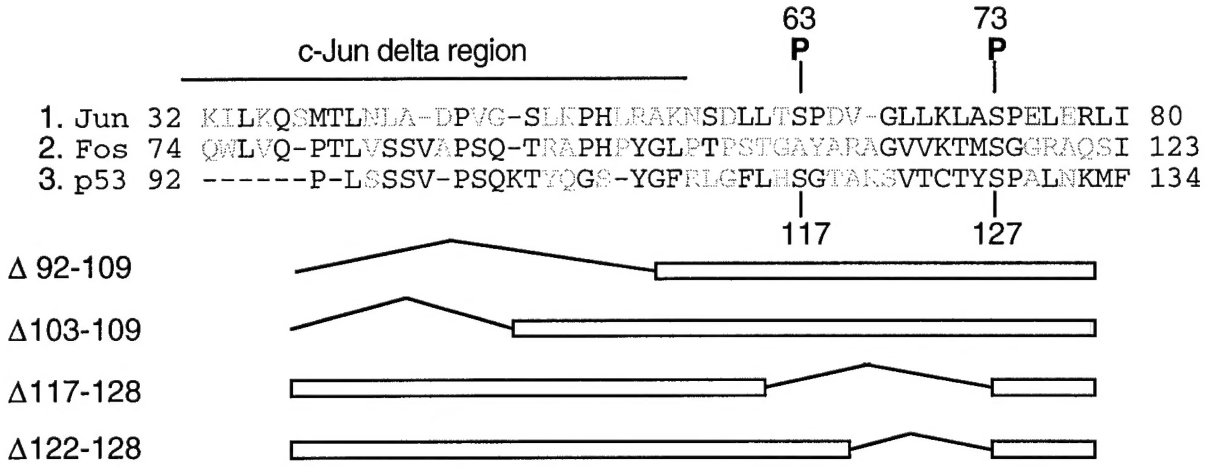


Figure 1. p53 contains an inhibitory domain. *In vivo* transactivation of G5E1B CAT gene by Gal4-p53 fusion proteins in transient transfection assays. CAT activity quantified with an AMBIS b-scanning system is represented as a percentage of the activity with Gal4-VP16.

Similar results have been observed in the c-jun and c-fos proteins, both of which contain conserved inhibitory domains capable of silencing their activation domains. Furthermore, the δ region of the c-jun inhibitory domain has been shown to interact with the c-jun N-terminal kinase (JNK). JNK is a member of the mitogenic activated protein kinase (MAPK) family which can positively regulate c-jun activity through phosphorylation of serine residues 63 and 73. A

comparison of the regulatory regions of c-jun and c-fos has identified homology with a p53 inhibitory domain (92-160, see below). It is interesting to note that the proposed p53 regulatory region also contains two conserved serine residues at position 117 and 127 which may also be phosphorylated by JNK or a JNK related kinase. Therefore, we proposed to characterize JNK or a JNK related kinase.



RESULTS

During the grant support period (7/97 to 6/00), we constructed a set of deletions to disrupt the potential regulatory domains of p53 on Gal4-p53(1-160) and on full-length p53 and obtained the following results:

Mutants Gal4-p53N(Δ92-109), Gal4-p53N(Δ117-128) and Gal4-p53N(Ser117/127Ala) had little effect on the putative inhibitory effect

We constructed a set of mutants, Gal4-p53N(Δ92-109), Gal4-p53N(Δ117-128) and Gal4-p53N(Ser117/127Ala), and examined their ability to activate transcription in vivo. This was performed using a transient transfection assay in COS-7 cells in which Gal4-p53 fusion proteins are tested for their ability to stimulate expression of luciferase gene under control of a promoter containing five Gal4 binding sites upstream of a TATA box. Gal4-p53N and Gal4-p53(1-92) were used as controls. Our result reveals that low levels of transcriptional activity of the mutant Gal4-p53N which are similar to Gal4-p53N, suggesting that mutants Gal4-p53N(Δ92-109), Gal4-p53N(Δ117-128) and Gal4-p53N(Ser117/127Ala) had little effect on the putative inhibition (Figure 1).

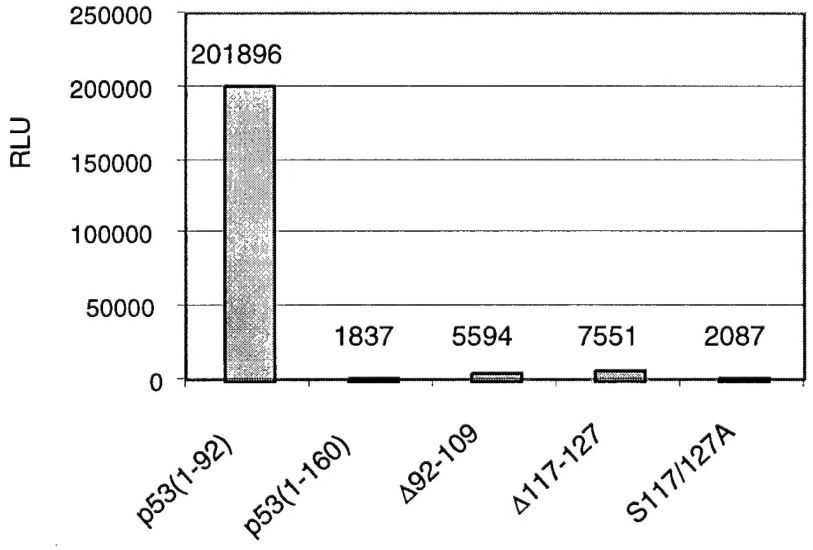


Figure 2. Transcriptional activity of Gal4-p53 constructs measure with a luciferase reporter plasmid in transfected COS-7 cells.

TPA stimulation inhibited the transcriptional activities of Gal4-p53N(Δ92-109) and Gal4-p53N(Δ117-128), but did not affect Gal4-p53(1-92).

We consider the possibility that failure to detect any effect for the mutants may be resulted from a lack of JNK or JNK related kinase activity in the cells. To test this, we stimulate the activity of JNK with TPA in NIH3T3 cells at different times after transfection. Our results show that stimulation by TPA reduced transcriptional activity of Gal4-p53N(Δ92-109) and Gal4-p53N(Δ117-128), but did not affect Gal4-p53(1-92), suggesting that residues within 92-109 and 117-128 domains may play a role to reduce p53 transcription activity in response to TPA stimulation. However, even with TPA stimulation, no significant effect on the inhibition was observed with deletion mutant Δ92-109 and Δ117-127.

Table I

	NO TPA	TPA STIMULATION
E4TATA-Luc	136	0
Gal4-p53N	0	0
p53N(1-92)	4236	5679
p53N(Δ92-109)	906	388
p53N(Δ117-127)	663	184
Gal4-VP16	83360	127590

Mutants p53(Δ92-109), p53(Δ117-128), p53(S117/127A) and p53(S117/127D) significantly lost their ability to activate transcription in vivo.

To study the putative inhibitory domain, we also constructed a set of mutants, p53(Δ92-109), p53(Δ117-128) and p53(S117/127A) on full-length p53, and examined their ability to activate transcription in vivo. This was performed by using a transient transfection assay in SAO-S2 cells in which p53 proteins are tested for their ability to stimulate expression of luciferase gene under control of a promoter containing five p53 binding sites upstream of a TATA box. Results of representative luciferase assay are shown in Figure 3. This result reveals that mutants p53(Δ92-109), p53(Δ117-128), p53(S117/127A) and p53(S117/127D) lost their transcription activity.

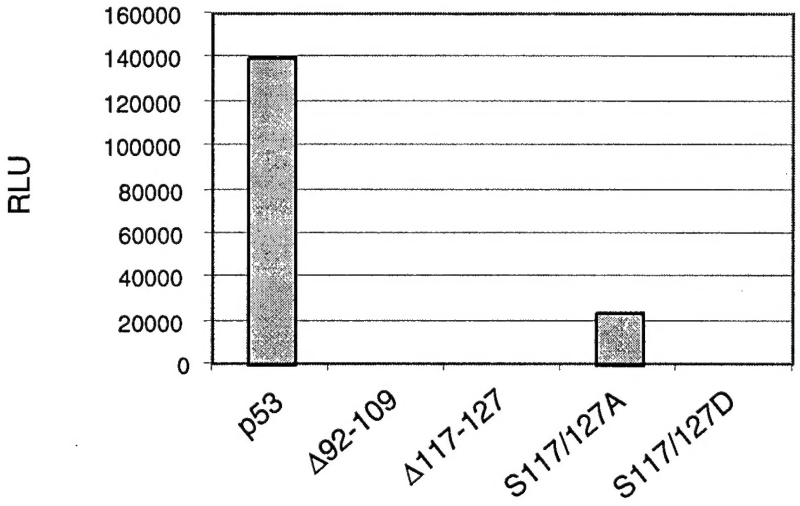


Figure 3. Transcriptional activity of pcDNAp53 constructs measured using a luciferase reporter plasmid in transfected SaoS-2 cells.

Wild-type p53 is rapidly degraded in part through the ubiquitin pathway. We consider the possibility that failure to detect any transcription activity may be caused by a decrease in p53 protein levels. To test this, we examined the p53 protein levels in Sao-S2 cells transiently transfected with wild-type p53 and mutant constructs. Results of a representative Western blot analysis are shown in Figure 4. Surprisingly, mutants p53(Δ92-109), p53(Δ117-128) and p53(S117/127D) displayed increased protein levels despite their defect in transcription. Our results also showed that p53(S117/127A) showed decreased protein levels in agreement to its ability to stimulate transcription, suggesting that Ser117/127 may play a role in stabilizing p53.

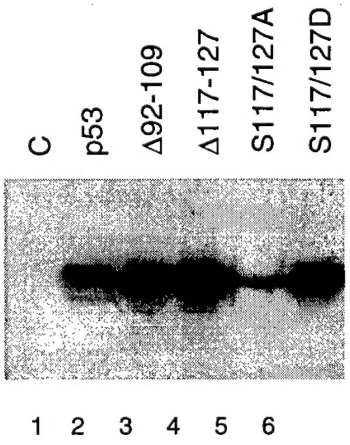


Figure 4. p53(S117/127A) displayes a decrease in protein levels .
Western blot analysis shows that the p53 protein levels in Sao-S2 cells transiently transfected with control, wild-type p53 and mutant constructs
as indicated.

Mutants p53(Δ92-109), p53(Δ117-128) and p53(S117/127A) retained their abilities to bind to DNA, but p53(S117/127D) not.

We also consider the possibility that deletion mutants may affect p53 DNA-binding activity. To test this, a gel shift experiment was performed. This was done by using nuclear extract from Sao-S2 cells transiently transfected with wild-type p53 and mutant constructs. Results of a representative gel-shift assay are shown in Figure 5. These results showed that the mutants p53(Δ92-109), p53(Δ117-128) and p53(S117/127A), but not p53(S117/127D), retained their abilities to bind to DNA, suggesting that the loss of the transcription activity may not be resulted from DNA-binding.

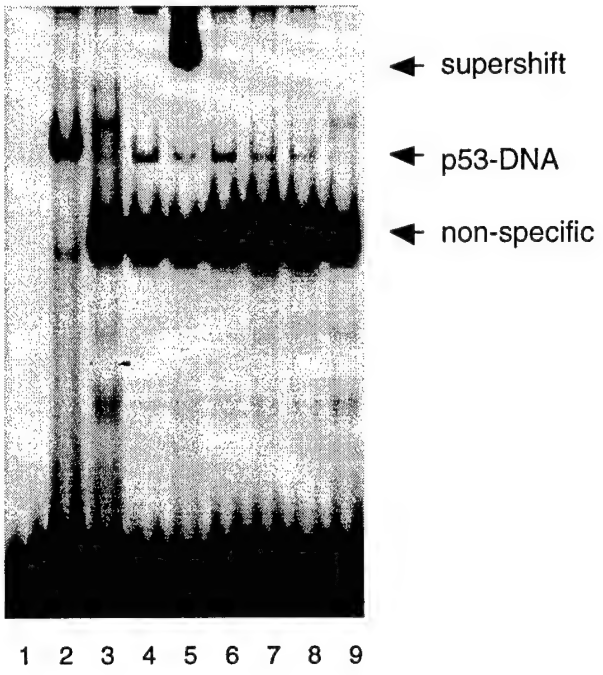


Figure 5. DNA-binding activities of mutant p53. A radiolabeled probe containing the p53-binding site from RGC was incubated with nuclear extracts from Sao-S2 cells transfected with wild-type p53 and mutant constructs as indicated. Lane 1 represents RGC probe, lane 2, 50 ng of purified p53, lane 3, transfected with control DNA, lane 4, transfected with wil-type p53, lane 5, lane 4 supershifted with anti-p53 antibody, lanes 6 to 9, transfected with Δ92, Δ117-127, S117/127A and S117/127D.

MAP-kinase is involved in the degradation of mutant p53 protein (Song et al, JBC)

Although we have failed to identify JNK or its related kinase as a kinase for wild-type p53, we have showed that MAP-kinase is involved in the degradation of mutant p53 protein. Overexpression of mutant p53 has been reported to promote tumorigenicity in several cancers. However, despite its potential importance, the signals regulating mutant p53 protein expression are not known. We have shown that a mutated form of p53 that is incapable of binding DNA is overexpressed in the acute promyelocytic leukemia NB4 cell line. Our results demonstrate that treatment of NB4 cells with bryostatin-1, which induces differentiation in this cell line, leads to

hyperphosphorylation of this DNA-binding impaired form of p53 via mitogen-activated protein kinase (MAPK). Following this phosphorylation, the p53 protein is degraded by the ubiquitin/proteasome pathway. Furthermore, we show that inhibition of p53 hyperphosphorylation by MAPK blocks p53 protein degradation and cell differentiation. In addition, inhibition of the ubiquitin/proteasome pathway also blocks p53 protein degradation and cell differentiation. These findings suggest a role for MAPK in the degradation of the DNA-binding impaired form of p53 protein and in the bryostatin-induced differentiation observed in this cell line. These results indicate the functional significance of p53 phosphorylation and degradation in cell differentiation.

KEY RESEARCH ACCOMPLISHMENTS

- MAPK is involved in regulating p53 transcription activity (Song et al, JBC 274:1677-1682)
- Serine residues 117 and 127 may play a role in stabilization of p53 protein levels

REPORTABLE OUTCOMES (PUBLICATIONS)

Song, X.D., H.M. Sheppard, A.W. Norman and X. Liu. 1999. MAP-kinase is involved in the degradation of p53 protein in the bryostatin-1 induced differentiation of the acute promyelocytic leukemia cell line NB4. *J. Biol. Chem.* **274**:1677-1682.

CONCLUSIONS

We have shown that two serine residues 117 and 127 may play a role in stabilization of p53 protein levels. Further experiments will be conducted to confirm these results.

We have showed that MAP-kinase is involved in the degradation of mutant p53 protein. Our findings suggest a role for MAPK in the degradation of mutated form of p53 protein and in

cell differentiation. These results were published as a JBC paper (Song, XD, H.M. Sheppard, A.W. Norman and X. Liu. 1999. Mitogen-activated protein kinase is involved in the degradation of p53 protein in the bryostatin-1 induced differentiation of the acute promyelocytic leukemia cell line NB4. J. Biol. Chem. 274:1677-1682).

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Mitogen-activated Protein Kinase Is Involved in the Degradation of p53 Protein in the Bryostatin-1-induced Differentiation of the Acute Promyelocytic Leukemia NB4 Cell Line*

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Overexpression of mutant p53 has been reported to promote tumorigenicity in several cancers. However, despite its potential importance, the signals regulating mutant p53 protein expression are not known. Here we show that a form of p53 that is incapable of binding DNA is overexpressed in the acute promyelocytic leukemia NB4 cell line. Our results demonstrate that treatment of NB4 cells with bryostatin-1, which induces differentiation in this cell line, leads to hyperphosphorylation of this DNA binding-impaired form of p53 via mitogen-activated protein kinase. After this phosphorylation, the p53 protein is degraded by the ubiquitin/proteasome pathway. Furthermore, we show that inhibition of p53 hyperphosphorylation blocks p53 protein degradation and cell differentiation. In addition, inhibition of the ubiquitin/proteasome pathway also blocks p53 protein degradation and cell differentiation. These findings suggest a role for mitogen-activated protein kinase in the degradation of the DNA binding-impaired form of p53 protein and in the bryostatin-induced differentiation observed in this cell line. The implications of these results with respect to the functional significance of p53 phosphorylation and degradation in cell differentiation are discussed.

Studies of human and mouse p53 have shown that wild-type p53 exerts its antiproliferation function by inducing growth arrest and apoptosis, whereas mutant p53 loses this function (1, 2). The biochemical activity of p53 that is required for this relies on its ability to bind to specific DNA sequences and to function as a transcription factor (3). The importance of the activation of transcription by p53 is underscored by the fact that the majority of p53 mutations found in tumors are located within the domain required for sequence-specific DNA binding (1, 2). Therefore, it is clear that this activity is critical to the role of p53 in preventing proliferation. Although the precise molecular mechanisms by which mutant p53 loses its antiproliferation functions remain to be elucidated, three models have been proposed (1). First, mutations in p53 may result in a loss of tumor suppressor function. Second, mutant p53 may have a dominant negative effect over wild-type p53 activity. Finally, mutations in p53 may lead to "a gain of function" such that it can induce proliferation and promote tumorigenicity of various cells (4–6).

An important mechanism used to control p53 activity is the regulation of p53 protein levels. Regulation is primarily achieved via protein degradation, although p53 levels may also be controlled at the level of transcription (7) and translation (8). Studies with human papilloma virus E6 protein and cellular oncogene Mdm2, which interact with and lead to the degradation of p53, have revealed that p53 is degraded by the ubiquitin-mediated proteolytic pathway (9–11). Although signals that target p53 for degradation are not yet fully understood, it is generally accepted that the phosphorylation status of p53 may be involved. As an illustration of this, cells treated with the serine phosphatase inhibitor okadaic acid accumulate high levels of hyperphosphorylated wild-type p53 (12). Clearly, the regulation of p53 protein level is important to its tumor suppressor function. Therefore, it follows that the regulation of mutant p53 protein levels may be important in regulating the oncogenic potential of mutant p53.

In this study we examined the involvement of the p53 pathway in NB4 cell differentiation. The NB4 cell line was originally isolated from a patient with acute promyelocytic leukemia and is characterized by a translocation involving chromosomes 15 and 17 (13). It has been used as a model for studying the mechanisms of cell differentiation, as it can be terminally differentiated into either mature neutrophilic granulocytes (14) or monocyte/macrophage-like cells (15) in response to various treatments. Among the treatments that have been shown to induce NB4 cells to differentiate into monocytes/macrophage-like cells are 1α,25-dihydroxyvitamin D_3 (16) and bryostatin-1 (17). Although the precise mechanisms involved in this differentiation are not yet fully understood, it is clear that a mitogen-activated protein kinase (MAPK)[1] pathway is involved (17, 18). Interestingly, MAPK has been shown to phosphorylate the amino terminus of p53 *in vitro* (19). Thus, a possible mechanism for bryostatin-induced differentiation may involve the phosphorylation of p53 via the MAPK pathway, resulting in an alteration of p53 antiproliferation activity.

To address the question of whether the p53 pathway is involved in bryostatin-induced differentiation, we asked whether endogenous p53 is phosphorylated in response to bryostatin treatment. Our results show that p53 becomes hyperphosphorylated and that the p53 protein is degraded via the ubiquitin/proteasome pathway after treatment with bryostatin-1. Furthermore, we demonstrate that inhibition of p53 hyperphosphorylation by a MAPK pathway inhibitor, PD98059, blocks p53 protein degradation and cell differentiation. In addition, inhibition of p53 protein degradation also blocks cell differentiation. The correlation between these effects suggests a role for MAPK in p53 degradation and in the bryostatin-

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; EMSA, electrophoresis mobility shift analysis; PAGE, polyacrylamide gel electrophoresis.

induced differentiation in this cell line. To assess the physiological significance of these observations, we examined the DNA binding ability of p53 purified from NB4 cells using anti-p53 antibody, Pab 421. The purified p53 was incapable of binding DNA in electrophoresis mobility shift analysis (EMSA), indicating that a mutant form of p53 exists in NB4 cells. These results suggest that following bryostatin treatment of NB4 cells, the mutant form of p53 is phosphorylated via the MAPK pathway and subsequently degraded. The implication of these observations with respect to the functional significance of p53 phosphorylation and degradation in cell differentiation are discussed.

EXPERIMENTAL PROCEDURES

Cell Culture and Protein Purification—NB4 cells were cultured in Dulbecco's modified Eagle's medium/F-12 media supplemented with 10% fetal bovine serum. The cells were routinely grown as suspension cultures, and passages 8 to 20 were used for each assay. Bryostatin-1 (Alexis, CA) was dissolved in ethanol. PD98095 (Calbiochem) and MG132 (Calbiochem) were dissolved in Me_2SO.

p53 was immunopurified from nuclear extracts prepared from NB4 cells according to the method of Dignam *et al.* (20). One milliliter of nuclear extract (7 mg of protein/ml) was incubated for 3 h at 4 °C with gentle rotation with 100 μl of packed protein A-Sepharose beads covalently coupled with anti-p53 antibody Pab 421. Beads were washed twice with 0.5 M KCl D buffer (20 mM HEPES, pH 7.9, 20% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) and once with 0.1 M KCl D buffer. p53 was eluted from the washed beads with 100 μl of 421 epitope oligopeptide (KKGQSTSRHKK) at 1 mg/ml concentration in 0.1 M KCl D buffer. Recombinant p53 was prepared from HeLa cells infected with recombinant vaccinia virus expressing p53 (21) as described above. Proteins were analyzed by SDS-PAGE followed by Western analysis or silver-staining to visualize bands.

Detection of p53 Phosphorylation and Protein Level in NB4 Cells—Two ml of NB4 cells at 10^6 cell/ml were labeled with [^{32}P]orthophosphate (ICN) in phosphate-deficient Dulbecco's modified Eagle's medium (Life Technologies, Inc.) and then incubated for 1 h followed immediately by bryostatin-1 treatment. At the end of the treatment, the cells were washed with PBS containing 100 μM vanadate and lysed by adding 0.5 ml of radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2 mM Na_3VO_4, 2 mM EGTA, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.25% sodium deoxycholate, 1% Nonidet P-40, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 2 μg/ml pepstatin). The lysate was homogenized by a Dounce homogenizer and clarified by centrifugation at 14,000 × *g* for 15 min at 4 °C. The supernatant were then incubated overnight at 4 °C with 20 μl of packed protein A-Sepharose beads to which Pab 421, an antibody specific for p53, was covalently linked. The immunoprecipitate was subjected to SDS-PAGE, and the labeled proteins were visualized with a PhosphorImager using Adobe Photoshop software. To determine p53 protein levels, NB4 cells were lysed with radioimmune precipitation buffer as described above, and the protein concentration for each sample was measured. Equivalent amounts of cell lysate were analyzed by SDS-PAGE followed by Western blot analysis using Pab 421.

Detection of Activated MAPK—Phosphorylated MAPK was detected by immunoprecipitation with anti-phosphotyrosine monoclonal antibody followed by immunoblotting using anti-MAP kinase antibody as described (18). Briefly, 2 × 10^6 NB4 cells were lysed with 0.5 ml of radioimmune precipitation buffer, and protein concentration was determined with a protein assay kit (Bio-Rad). Supernatant containing an equivalent amount of protein from each sample was incubated with 20 μl of packed agarose beads coupled to a monoclonal anti-phosphotyrosine antibody (PT-66, Sigma) at 4 °C overnight. The immunoprecipitate was then analyzed by SDS-PAGE followed by Western analysis with a rabbit anti-p42 MAPK polyclonal antibody (Pab C14, Santa Cruz). Equal loading of MAP kinase protein was determined by Western blot analysis using anti-p42 MAPK antibody.

EMSA—An oligonucleotide probe containing the ribosomal gene cluster (RGC) p53-binding site was used containing the sequence 5′-AGCTTGCCTCGAGCTTGCCTGGACTTGCCTGGTCGACGC-3′. Binding reactions contained 60 mM KCl, 12% glycerol, 5 mM $MgCl_2$, 1 mM EDTA, 10 μg bovine serum albumin, 0.2 μg of poly(d(G-C)), 600 cpm of ^{32}P-labeled probe and proteins as indicated, and water in a total volume of 12.5 μl. Reactions were incubated for 30 min at 30 °C and then analyzed on a 3% polyacrylamide gel containing 0.5 × TBE (0.045 mM Tris borate, 0.045 mM sodium borate, 0.001 mM EDTA, pH 8.0). The gel was dried, and DNA-protein complexes were visualized with a PhosphorImager using Adobe Photoshop software.

TABLE I
Effect of PD98059 and MG132 on bryostatin-induced cell differentiation

Treatments	Adherence	Phagocytosis
	%	%
Control	0.2	0
Bryostatin-1	35	21
PD98059	0.2	0
Bryostatin + PD	3	2
MG132	0.2	0
Bryostatin + MG	0.2	0

Northern Blot Analysis—Total RNA was isolated using TRIzol reagent (Life Technologies, Inc.) according to the manufacturer's instructions. Thirty μg of total RNA was subjected to electrophoresis in a 1.5% agarose gel and transferred to a MAGNA nylon transfer membrane (Micron Separations Inc.). A 1.3-kilobase pair DNA fragment corresponding to the full-length human p53 gene was cut from plasmid pcDNA-p53 (22) and labeled using T7 QuickPrime Kit (Amersham Pharmacia Biotech) as a probe. Northern analysis was conducted using a standard procedure (23). The RNA bands were visualized with a PhosphorImager using Adobe Photoshop software. The membrane was then stripped and hybridized with a glyceraldehyde 3-phosphate dehydrogenase cDNA probe to normalize for RNA loading.

Studies of Cell Differentiation—To examine the effect of bryostatin-1 on cell adherence, 2 × 10^4 cells were seeded in a 24-well plate. Cells were pretreated with Me_2SO control or 5 μM PD98059 or 1 μM MG132 for 30 min and then treated with vehicle or 5.6 nM bryostatin-1 for 72 h. At the end of the treatment, cells in suspension *versus* those adhered to the culture plate were counted. A total of 1000 cells were counted for each treatment, and adherence was expressed as a percentage of adherent cells to the total number of cells. Phagocytosis was measured by incubation of NB4 cells with latex beads (3 μm, Sigma) for 5 h after 72 h treatment of cells with the reagents as described above. Cells were gently washed 4 times and centrifuged at 125 × *g* for 5 min to remove the free beads. A total of 500 cells were counted for each treatment, and phagocytosis was expressed as the percentage of bead-engulfing cells to total cells. Both bead-engulfing cells and total cells were counted under a microscope and photographed.

RESULTS

p53 Hyperphosphorylation and Reduction in NB4 Cell Differentiation—To determine whether the p53 pathway is involved in NB4 cell differentiation, we first investigated whether the phosphorylation status of p53 was altered in response to bryostatin treatment. NB4 cells have been characterized with respect to differentiation induced by bryostatin-1 (Ref. 17; also see Fig. 5 and Table I). Initial experiments suggested that treatment with 5.6 and 56 nM bryostatin-1 induced differentiation. Therefore, NB4 cells were metabolically labeled with $^{32}P_i$ and immediately treated with 0, 0.56, 5.6, or 56 nM bryostatin-1. From each treatment, lysate containing equivalent amounts of protein was immunoprecipitated with anti-p53 antibody. After electrophoresis, the amount of phosphorylation of p53 was analyzed by autoradiography (Fig. 1*A*). In the absence of bryostatin-1, p53 protein showed basal levels of phosphorylation, which is as expected for a phosphoprotein. The addition of bryostatin-1, however, resulted in an increase in phosphorylation above this basal level. The maximum level of phosphorylation was observed after treatment with 5.6 and 56 nM bryostatin-1 (Fig. 1*A*). It was also demonstrated that the maximum level of phosphorylation was obtained 30 min after bryostatin treatment (Fig. 1*B*). A control Western analysis was performed to ensure the equivalent amounts of p53 protein present in each sample (data not shown). Consequently, these results indicate that p53 becomes hyperphosphorylated after bryostatin treatment in a dose- and time-dependent manner.

Next we asked whether bryostatin-1 would affect p53 protein levels, as it has been previously suggested that the phospho-

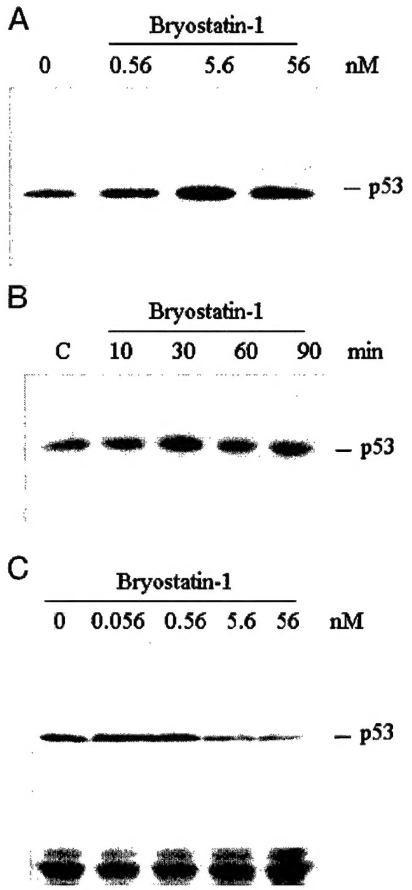

FIG. 1. **Treatment with bryostatin-1 induces p53 protein phosphorylation and reduction in NB4 cells.** NB4 cells were labeled with [^{32}P]orthophosphate and were treated with control or with bryostatin-1 at various concentrations as indicated for 30 min (*A*) or treated with 5.6 nM bryostatin-1 for various time periods as indicated (*B*). Cells extracts were then prepared, and p53 protein was immunoprecipitated using p53 antibody. Protein samples were resolved by electrophoresis on a SDS-PAGE gel and visualized by autoradiography. *C*, to study p53 protein reduction NB4, cells were treated with various concentrations of bryostatin-1 as indicated for 12 h. Cells extracts were then prepared, and Western blot analysis was performed with anti-p53 antibody (*top panel*). Equal loading of protein from crude extract was verified by Coomassie Blue staining (*bottom panel*).

rylation status of p53 may be involved in its targeting for degradation. The NB4 cells were treated with increasing amounts of bryostatin-1 or control vehicle, and the resulting p53 protein level was analyzed by Western analysis (Fig. 1*C*). It was anticipated that the hyperphosphorylation of p53 would result in an increase in p53 protein levels as phosphatase inhibitor treatment results in an accumulation of high levels of wild-type p53 (12). Surprisingly, however, treatment of cells with 5.6 and 56 nM brystatin-1 significantly reduced p53 protein levels. Treatment with 0.056 and 0.56 nM bryostatin-1 had little effect. Coomassie Blue staining of major representative bands indicated that equivalent amounts of protein were present (Fig. 1*C*, *lower panel*). These results suggest that treatment with bryostatin-1 decreases p53 protein levels in NB4 cells. Furthermore, the results indicate that the concentrations of bryostatin-1 sufficient to increase p53 phosphorylation (5.6 and 56 nM) are the same as those required to decrease p53 protein levels. Similarly, those bryostatin concentrations that had little effect on p53 phosphorylation were also not effective in reducing the protein levels. We interpret this correlation to indicate that hyperphosphorylation of p53 might be involved in the reduction of p53 protein levels. It is particularly interesting to note that treatment of NB4 cells with bryostatin-1 at concentrations of 5.6 and 56 nM but not at 0.56 nM has been shown to induce differentiation (Fig. 5 and Table I).[2]

MAPK Pathway Is Involved in p53 Hyperphosphorylation and Reduction in p53 Protein Levels—We have shown that MAPK is activated in NB4 cells after bryostatin treatment,[2] which raised the possibility that the MAPK pathway might be involved in p53 hyperphosphorylation and protein reduction. To address this question, a specific MAPK pathway inhibitor, PD98059 (24), was employed. Phosphorylation of MAPK can be blocked in a dose-dependent manner by this inhibitor, but it is maximally effective at a concentration of 5 μM. Five min after treating NB4 cells with 5.6 nM bryostatin-1, phosphorylation of MAPK (p44 and p42) was significantly increased in the absence of inhibitor (Fig. 2*A*). When 5 μM PD98059 was included with the bryostatin-1 treatment, no detectable increase in MAPK phosphorylation was found. This result confirms that PD98059 can block bryostatin-induced MAPK activation in this cell line. We next examined whether PD98059 was able to block p53 hyperphosphorylation and protein reduction. Fig. 2*B* shows that treatment with 5.6 nM bryostatin-1 increased phosphorylation of p53 and that concurrent treatment with PD98059 blocked this bryostatin-induced p53 hyperphosphorylation in a dose-dependent manner. This is consistent with the hypothesis that MAPK is involved in the bryostatin-induced phosphorylation of p53. Importantly, treatment with PD98059 also inhibited bryostatin-induced p53 protein reduction (Fig. 2*C*). Coomassie Blue staining of major representative bands indicated that equivalent amounts of protein were present (Fig. 2*C*, *lower panel*). Consequently, our data demonstrate that the MAPK pathway is involved in p53 hyperphosphorylation and in the reduction in p53 protein levels. Although direct phosphorylation of p53 by the MAPK pathway remains to be elucidated, it is clear that MAPK is involved. In addition, these results suggest that p53 hyperphosphorylation might be associated with a reduction in p53 protein levels.

Reduction in p53 Protein Levels Is Mediated by Ubiquitin/Proteasome Pathway—We next asked if the reduction in p53 protein levels was because of protein degradation. To address this question, we examined the steady state level of p53 mRNA by Northern analysis. NB4 cells were treated with either 5.6 nM brystatin-1 or control vehicle, and mRNA was extracted at various time points as indicated (Fig. 3, *top*). As a control to ensure equal loading of RNA, a probe specific for glyceraldehyde 3-phosphate dehydrogenase was used (Fig. 3, *bottom*). Our results demonstrate that the reduction in p53 protein levels was not caused by a decrease in p53 mRNA levels. This implied that the reduction in p53 protein levels might be caused by a decrease in protein stability.

To test this hypothesis, sodium borohydride ($NaBH_4$), an inhibitor of ubiquitin COOH-terminal hydrolase, was used to determine whether an inhibition of the ubiquitin/proteasome pathway would block the bryostatin-induced decrease in p53 protein levels. Ubiquitin COOH-terminal hydrolase is required for the generation of the functional monomeric form of ubiquitin (25) and is suggested to play a role in bryostatin-induced Reh cell differentiation (26). Our results show that bryostatin-induced p53 reduction was completely inhibited by sodium borohydride (Fig. 3*B*). As proteasomes play a key role in protein degradation, we then tested whether a proteasome inhibitor, MG132, could inhibit p53 degradation. Fig. 3*B* shows that, like sodium borohydride, MG132 also blocks bryostatin-induced p53 reduction. Taken together, these results suggest that the MAPK-mediated decrease in p53 protein levels is not caused by decreased gene transcription but by ubiquitin/proteasome-de-

[2] X-D. Song and A. W. Norman, unpublished data.

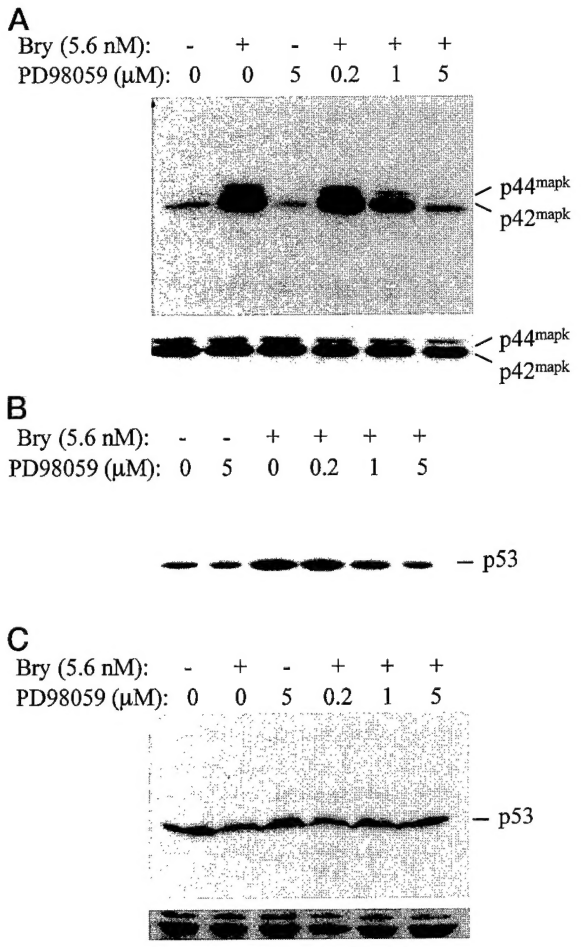

FIG. 2. **MAP kinase is involved in p53 phosphorylation and degradation in NB4 cells.** *A*, after a 30-min preincubation in the presence or absence of MAPK pathway inhibitor PD98059, NB4 cells were treated with control or 5.6 nM bryostatin-1 (*Bry*) for 5 min. Cells extracts were then prepared, tyrosine-phosphorylated proteins were immunoprecipitated with anti-phosphotyrosine antibody, and protein samples were resolved by electrophoresis on a SDS-PAGE gel. Western blotting was then performed with anti-MAP kinase antibody (*top panel*). To determine total levels of MAPK protein present in the cells, Western blotting with anti-MAP kinase antibody was performed on cell extracts (*bottom panel*). *B*, NB4 cells were labeled with [^{32}P]orthophosphate, PD98059 was added at various concentrations as indicated, either in the presence or absence of 5.6 nM bryostatin-1, and incubation was continued for a further 30 min. Cells extracts were then prepared, and p53 protein was immunoprecipitated. Protein samples were resolved by electrophoresis on a SDS-PAGE gel and visualized by autoradiography. *C*, NB4 cells were preincubated with PD98059 at various concentrations as indicated, after which control or 5.6 nM bryostatin-1 was added, and incubation was continued for 12 h. Cells extracts were then prepared, and Western blotting was performed with anti-p53 antibody. Equal loading of protein from crude extract was verified by Coomassie Blue staining (*bottom panel*).

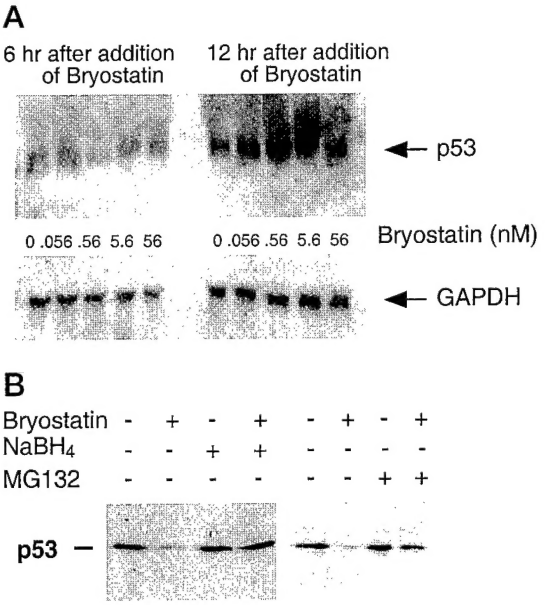

FIG. 3. **The reduction in p53 protein levels is because of protein degradation.** *A*, NB4 cells were treated with control vehicle or with bryostatin-1 at various concentrations as indicated for 6 or 12 h, after which total RNA was extracted. Thirty μg of total RNA from each sample were subjected to Northern blot analysis using a probe specific for p53 mRNA (*upper panel*). To ensure equal loading of RNA, the membrane was stripped and re-incubated with a probe specific for glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA *B*, to study the effect of inhibitors of the ubiquitin/proteosome pathway on bryostatin-1-induced p53 degradation, NB4 cells were preincubated with sodium borohydride ($NaBH_4$) or MG132 for 30 min, after which 5.6 nM bryostatin-1 was added, and incubation was continued for 12 h. Cells extracts were then prepared, and Western blotting was performed with anti-p53 antibody.

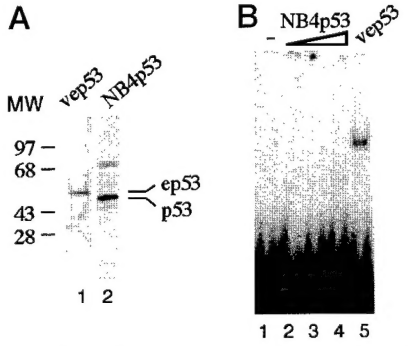

FIG. 4. **p53 purified from NB4 cells fails to bind to DNA in EMSA.** *A*, vaccinia virus expressed human p53 (vep53) purified from HeLa cell extracts and endogenous p53 purified from NB4 cell extracts were subjected to electrophoresis on a SDS-PAGE gel and visualized by silver staining. *B*, in EMSA, radiolabeled probe containing the p53 site from the ribosomal gene cluster was incubated with approximately 50, 75, or 100 ng of p53 purified from NB4 cells (*lanes 2–4*) or 20 ng of vep53 (*lane 5*) before electrophoresis on a 3% polyacrylamide gel. The position of vep53 and p53 expressed from the endogenous genes are shown. *MW*, molecular mass.

pendent protein degradation.

p53 from NB4 Cells Is Incapable of Binding DNA—In an effort to assess the functional significance of MAPK-mediated p53 phosphorylation and degradation in NB4 cell differentiation, we tested the ability of p53 purified from NB4 cells to bind DNA using EMSA. p53 was purified from nuclear extracts prepared from NB4 cells using anti-p53 antibody, Pab 421 (Fig. 4*A*, *lane 2*). This antibody recognizes an epitope in the carboxyl terminus of p53 and is thought to convert p53 from its latent to its active state and thereby significantly increase its DNA binding activity (27). Vaccinia virus expressed epitope-tagged human p53 purified from HeLa cells using the same antibody was used as a control (vep53, Fig. 4*A*, *lane 1*). When a DNA probe containing the p53 cis element identified in the ribosomal gene cluster was incubated with vep53, a shifted band was observed (Fig. 4*B*, *lane 5*). This band was supershifted by the addition of anti-p53 antibody N-19 (Santa Cruz), and the addition of a 100-fold excess of cold ribosomal gene cluster DNA fragment was sufficient to inhibit its formation, suggesting that this band was p53-specific (data not shown). In comparison to wild-type p53, p53 purified from NB4 cells had a significantly reduced affinity for DNA binding. The inability of this p53 to bind DNA suggests that either mutations exist within the protein or that the protein has been inactivated by post-

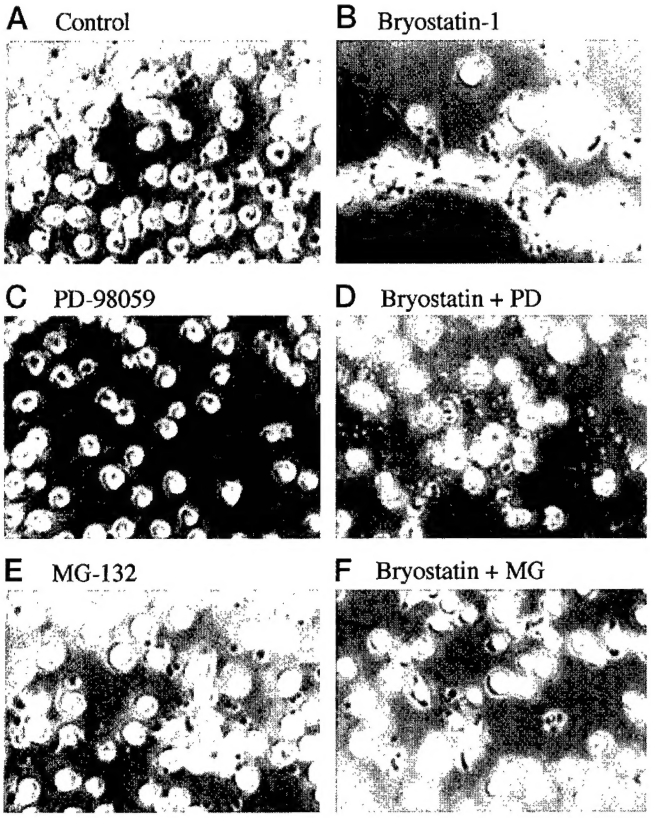

FIG. 5. **MAPK-mediated p53 degradation is associated with cell differentiation.** NB4 cells were treated with control (*A*), 5.6 nM bryostatin-1 (*B*), 5 μM PD98059 (*C*), bryostatin-1 and PD98059 (*D*), 1 μM MG132 (*E*), bryostatin-1 and MG132 (*F*) for 72 h. After each treatment, phagocytosis and cell adherence, the markers for NB4 cell differentiation, were measured by incubation of NB4 cells with latex beads for 5 h, and the cells were photographed with 400× magnification using a Nikon microscope.

translational modification. Regardless of the nature of the alteration, these results establish that p53 in NB4 cells is unable to bind DNA. It may be, therefore, that degradation of this DNA binding-impaired form of p53 is associated with NB4 cell differentiation.

MAPK-mediated p53 Degradation Is Associated with Bryostatin-induced NB4 Cell Differentiation—To demonstrate the physiological significance of the p53 degradation in NB4 cell differentiation, we tested whether the proteasome inhibitor, MG132, would block bryostatin-induced differentiation. Untreated NB4 cells grow as a suspension and contain nonadherent cells (Fig. 5*A*). When treated with 5.6 nM bryostatin-1, 35% of the cells become attached to the surface of the culture plate (Table I), and 21% exhibit a morphology related to monocyte/macrophages (Fig. 5*B*), which is the marker for NB4 cell differentiation. In contrast, concurrent treatment with MG132 blocked both the bryostatin-induced cell adherence (Table I) and phagocytotic activities (Fig. 5*F*), suggesting that inhibition of the ubiquitin/proteasome pathway blocked NB4 cell differentiation. Treatment with MG132 alone had little effect on NB4 cell differentiation (Fig. 5*E* and Table I) or cell viability (data not shown).

The observation that the MAPK pathway is involved in phosphorylation/degradation of a mutant form of p53 suggested that the MAPK pathway might be involved in cell differentiation. Therefore, we tested whether the MAPK pathway inhibitor, PD98059, could inhibit bryostatin-induced NB4 cell differentiation under the same conditions that were sufficient to induce p53 phosphorylation/degradation. Concurrent treatment of NB4 cells with bryostatin-1 and 5 μM PD98059 blocked the bryostatin-induced cell adherence (Table I) and phagocytotic activities (Fig. 5*D*), suggesting that inhibition of MAPK blocked NB4 cell differentiation. Treatment with 5 μM PD98059 alone had no effect on NB4 cell differentiation (Fig. 5*C* and Table I) or cell viability (data not shown). This observation supports the view that the MAPK pathway is involved in bryostatin-induced NB4 cell differentiation. The critical requirement for MAPK in differentiation coupled with the demonstration that MAPK is involved in phosphorylation/protein degradation of a mutant p53 suggests that degradation of mutant p53 may possibly play a role in cell differentiation.

DISCUSSION

A requisite first step toward understanding the molecular mechanism for cell differentiation and proliferation is to identify the signal transduction pathways and the cellular targets of those pathways involved in these processes. In this paper we present biochemical evidence that indicates that the MAPK pathway, required for bryostatin-induced cell differentiation, induces p53 hyperphosphorylation, which subsequently reduces p53 protein stability. This observation is particularly significant because an altered form of p53, unable to bind DNA *in vitro*, is overexpressed in NB4 cells. It has been suggested that mutant p53 may gain a function such that it can promote tumorigenicity in various cells. Consequently, degradation of mutant p53 protein may result in a reduction of tumorigenicity. Taken together, our results indicate that an altered form of p53 is hyperphosphorylated via the MAPK pathway and subsequently degraded. Although a direct cause and effect relationship is yet to be established, our findings indicate that a reduction in mutant p53 levels may contribute to cell differentiation.

Phosphorylation is one potential mechanism by which cells might regulate p53 protein levels and, hence, its antiproliferation activity. Experiments *in vivo* have clearly demonstrated that p53 is a phosphoprotein on which multiple phosphorylation sites have been identified (1). In comparison to wild-type p53, literature relating to the phosphorylation of mutant p53 and the control of its protein level is limited. In this study we provide *in vivo* evidence that a form of p53 which is incapable of binding DNA is hyperphosphorylated and subsequently degraded via the MAPK pathway, although it is not clear from these experiments whether MAPK phosphorylates this DNA binding-impaired form of p53 directly or whether additional kinases are required. Furthermore, our results suggest that phosphorylation of p53 leads to a reduction in protein stability mediated by the ubiquitin/proteasome pathway.

The observation of a reduction in protein stability after phosphorylation is in contrast to previous reports in which phosphorylation of p53 led to an increase in its stability (12, 28). However, in agreement with our findings, two proto-oncogene-encoded transcription factors, c-Jun and BCL-6, have also been shown to be degraded after phosphorylation by MAPK (29–30). Thus, degradation of oncogene products, including mutant p53, may represent a general mechanism by which the MAPK pathway controls cell function. Reduction of p53 protein levels was observed 12 h after bryostatin treatment, which is slower than the 2 h reported for BCL and c-Jun. It is possible that MAPK may mediate a second signal required for the reduction of p53 protein levels. Nevertheless, our data show that a strong correlation exists between p53 protein phosphorylation and stability.

The finding that mutant p53 is phosphorylated via the MAPK pathway and subsequently degraded has several implications when considering the model in which mutations in p53 lead to a gain of function that promotes tumorigenicity. First, overexpression of mutant p53 in pre-B (31), fibroblast (5), and osteosarcoma cells (32) has been shown to dramatically en-

hance the tumorigenicity of these cells. Reduction in p53 protein levels, therefore, may be of significance in preventing cell proliferation. Indeed, our data demonstrate a strong correlation between protein degradation and cell differentiation. Second, the high frequency of p53 mutations in human cancers warrants a detailed analysis of the molecular mechanisms of the gain of function of mutant p53 and the signals that may regulate this function. In this paper we go some way toward this goal by presenting evidence that the MAPK pathway is involved in degradation of the DNA binding-impaired form of p53 and by correlating this function with NB4 cell differentiation. Finally, a significant research effort is concerned with finding strategies to inactivate mutant p53. In light of the results presented here, it may also be effective to develop therapies designed to reduce mutant p53 protein levels.

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